SUPPLEMENTAL MATERIAL

Detailed Methods

Mouse Generation and Phenotypic Analyses

Drp1$^{loxp/loxp}$ mice [1] and Parkin$^{loxp/loxp}$ mice (obtained from Lexicon Pharmaceuticals [2]), individually and in combination, were bred with mice carrying a *myh6* promoter-driven nuclear-directed modified estrogen receptor (MER)-Cre transgene [3] to achieve tamoxifen-inducible gene deletion. Tamoxifen was administered to 8 week old adult mice as previously described [4]. Cardiomyocyte-specific human Parkin overexpression was achieved using the *myh6* promoter-driven doxycycline-suppressible ("tet-off") bi-transgeic system [5]; doxycycline was never administered to dames or pups, inducing transgene expression after birth [6]. M-mode echocardiography was performed on unsedated mice [4]; controls were age-matched Drp1$^{loxp/loxp}$ littermates, Drp1$^{loxp/loxp}$+Parkin $^{loxp/loxp}$ littermates with or without tamoxifen administration and tetracycline-controlled transactivator protein (tTA) transgenic mice. All experimental procedures were approved by the Washington University Institutional Animal Care and Use Committee.

RNA Expression Analysis

mRNA abundance of Parkin and associated mitophagy factors, mitochondrial dynamics factors, mitochondrial biogenesis factors, and representative cardiac-expressed genes were obtained by analyzing RNA sequence data on 25 normal adult FVB mouse hearts generated from this laboratory, available at NCBI GEO GSE55792 [7].

Parkin mRNA abundance was also measured by quantitative real-time PCR. Total RNAs were extracted from snap-frozen cardiac tissues with TRIzol (Invitrogen, 15596-026); single strand cDNA was generated using high-capacity cDNA reverse transcription kit with RNase inhibitor (Invitrogen, 4374966) following the manufacturer's protocol. RNA expression analysis was performed using Taqman qRT-PCR 2x master mix (Invitrogen, 4440038) with predesigned primer/probes sets for mouse Park2 (Mm00450187_m1) and GAPDH (Hs02758991_g1).

Western Blotting

Cardiac tissues were collected, snap-frozen in liquid nitrogen, and homogenized in either tissue extraction reagent (Invitrogen, FNN0071) or a homogenizing buffer (10 mM HEPES, 320 mM sucrose, 3 mM $MgCl_2$ and 1mM DTT), with protease inhibitor (Roche, 05892970001) and phosphatase inhibitor (Roche, 04906837001) as previously described [4]. Myocardial homogenates were collected from the supernatant after centrifugation at 3,800 x*g*, myocardial mitochondrial fractions were pelleted and resuspended after centrifugation at 10,000 x*g,* and myocardial cytosolic fractions were collected from the supernatant after centrifugation at 100,000 x*g*. Proteins were size-separated by 4-15% pre-cast gradient SDS-PAGE (Bio-Rad, 456-1084 and 456-1086), transferred to PVDF membranes (GE Healthcare, 10600021), and blocked with phosphate-buffered saline (PBS, Invitrogen, 70011-069) containing 0.1% Tween-20 (Promega, H5152) plus 5% nonfat dry milk (Bio-Rad, 170-6404) and/or 5% bovine serum albumin (BSA, Santa Cruz Biotech, 9048-46-8) at room temperature for 1 hour, incubated with primary antibodies at room temperature for 2 hours followed by incubation with horseradish peroxidase (HRP) conjugated secondary antibodies at room temperature for 1 hour, and visualized using the ECL chemiluminescence reagent (Bio-Rad, 170-5060).

Antibodies

For western blotting, primary antibodies against Drp1 (1:1000, ab56788), GAPDH (1:3000, ab8245), p62 (1:1000, ab56416), LC3 I/II (1:1000, ab128025), COX IV (1:1000, ab14744) were from Abcam. Primary antibody against ubiquitin (1:100, sc-8017) was from Santa Cruz Biotechnology. Primary antibody against Parkin was from Cell Signaling Technology (1:1000, #2132). Horseradish peroxidase (HRP)

conjugated secondary antibodies anti-mouse IgG (1:3000, cs7076) and anti-rabbit IgG (1:3000, cs7074) were from Cell Signaling Technology.

Histological Studies
Mouse heart tissues were fixed with 4% formaldehyde solution in PBS with or without prior transcardial perfusion. Paraffin-embedded tissues were sectioned at a thickness of 5 μm on a Leica RM2255 rotary microtome. The sections were de-paraffinized in xylene, rehydrated with a gradient (100-50%) of ethanol, and washed in distilled water. Masson's trichrome stain (Sigma, HT15, HT10132 & 34256) was performed according to the manufacturers' protocol. For Evans blue studies, mice were intraperitoneally administered 1% Evans blue dye (Sigma, E2129) solution in PBS at 1% volume relative (ml) to body mass (g) 20-24 h prior to tissue sampling [8]. The sections were stained with FITC conjugated-wheat germ agglutinin (Invitrogen, W834) at room temperature for 30 min before nuclear counterstaining with DAPI (Vector Laboratories, H-1200). Evans blue positive cardiomyocytes fluoresce in red.

Transmission Electron Microscopy
Mouse heart tissues were fixed with EM fixation buffer (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4). A Jeol electron microscope (JEM-1400, JEOL, Tokyo, Japan) at 800x - 8,000x direct magnifications was used for ultrastructural examination of osmium tetroxide/uranyl acetate stained mouse heart thin sections (90 nm). Mitochondrial content (% of the areas taken by mitochondria compared to those of the cardiomyocytes) and individual mitochondrial size and aspect ratio were measured using ImageJ (NIH) on transmission electron microscopic images.

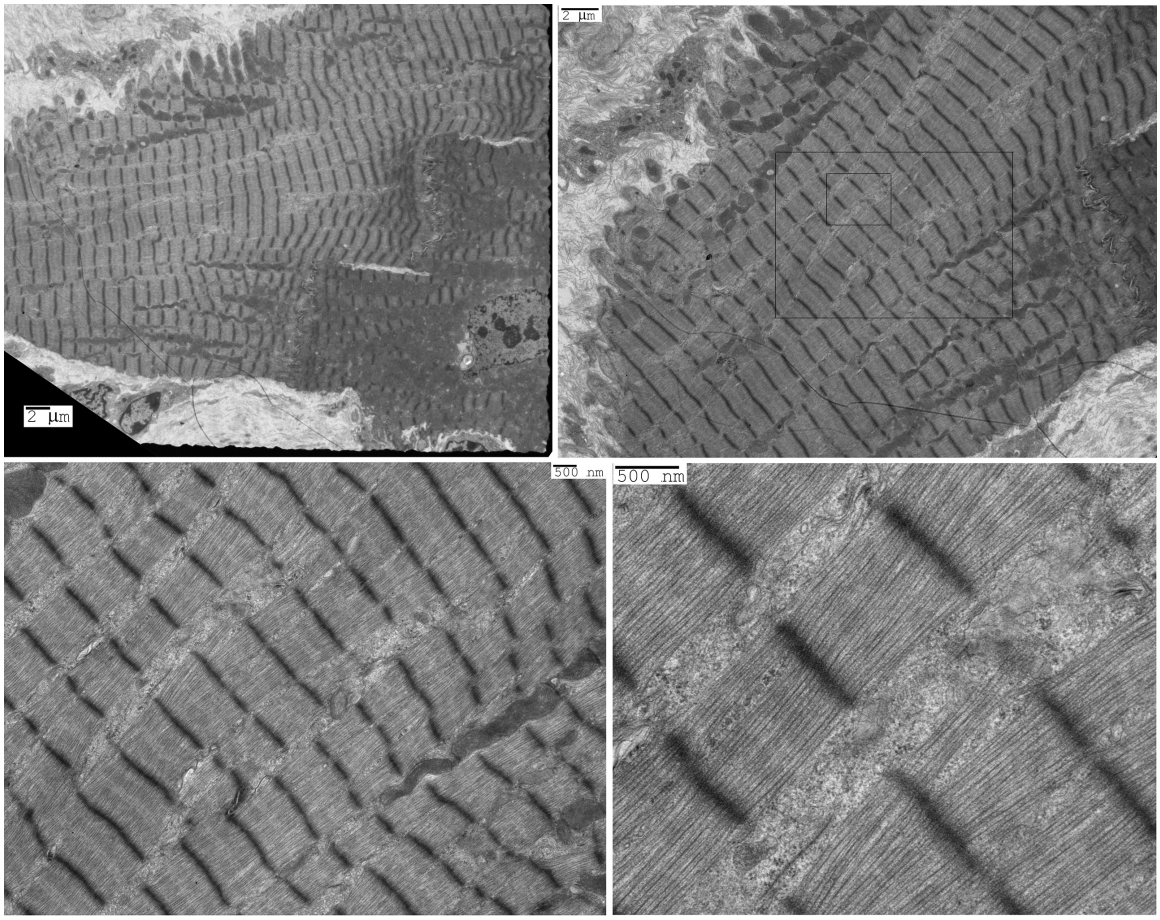
Isolation of Cardiac Mitochondria
Isolation of cardiac mitochondria used a modification of a published protocol [9]. Briefly, mouse hearts were minced, incubated with trypsin and then homogenized with a glass/teflon Potter Elvehjem homogenizer. The homogenates were centrifuged at 800 x*g* at 4 °C for 10 min. The supernatants were centrifuged at 8,000 x*g* at 4 °C for 10 min and the resulting supernatants were discarded. The pellet containing the mitochondria was washed and centrifuged at 8,000 x*g* at 4 °C for 10 min before resuspension for functional analyses. Mitochondrial protein concentration was colorimetrically measured using Bio-Rad protein assay dye reagent concentrate (Bio-Rad, 500-0006).

Mitochondrial Respiration
Respiratory activities of isolated mitochondria were assessed using a micro Clark-type electrode in a closed and magnetically stirred glass chamber as previously described [10]. Non-stimulated (-ADP) respiration (state 2) and ADP (Sigma, A2754)-stimulated respiration (state 3) were measured. Oligomycin (Sigma, 75351) was added to inhibit ATP synthesis.

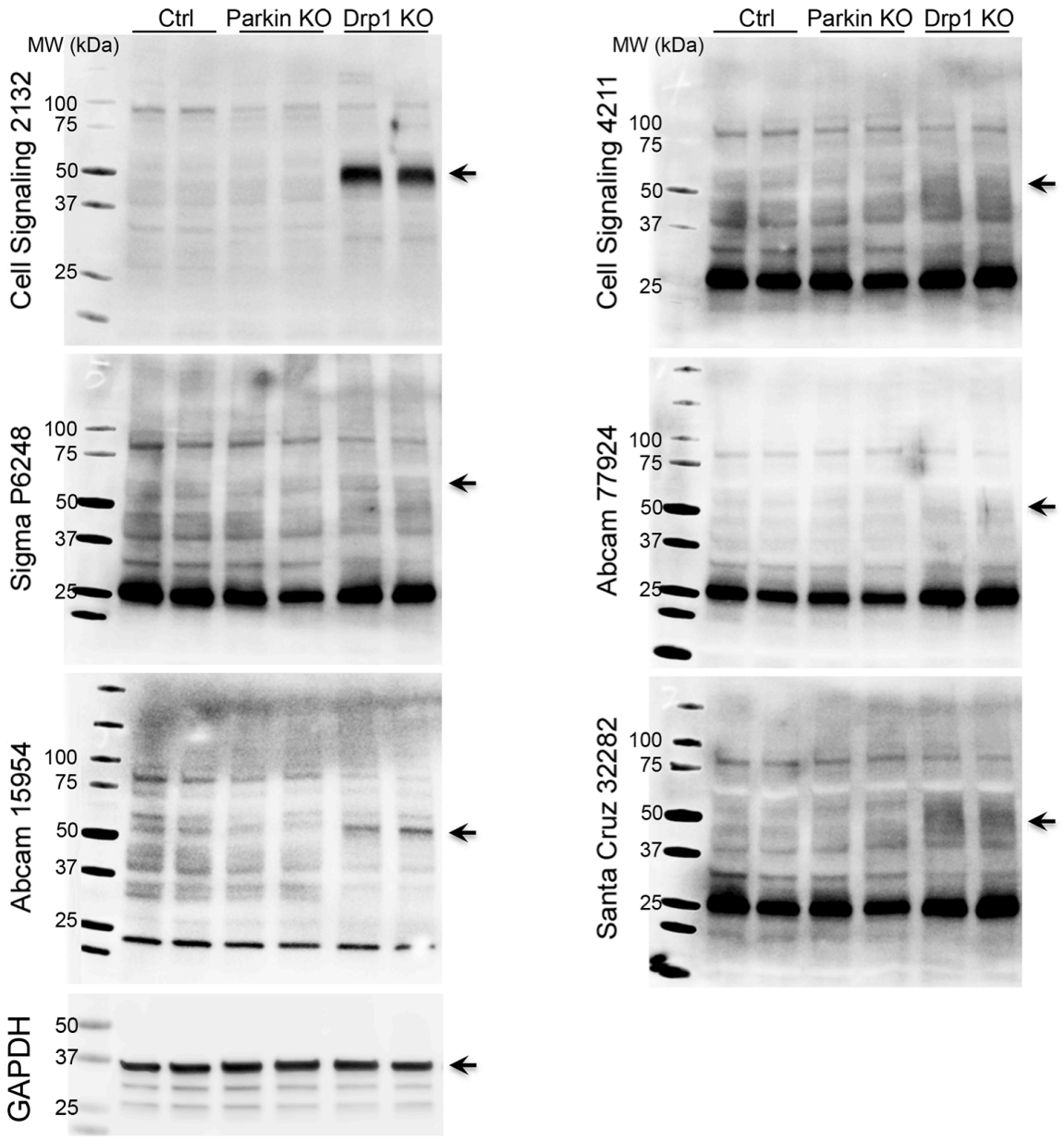
Flow Cytometric Analyses of Isolated Mitochondria
Isolated mitochondria were stained with 200 nM MitoTracker Green (Invitrogen, M-7514), 2 μM of tetramethylrhodamine, ethyl ester (TMRE, Invitrogen, T-669), or 2.5 μM MitoSOX red (Invitrogen, M36008) at room temperature for 20 min and washed twice with PBS. Flow cytometric analyses of mitochondrial size (forward scatter, FSC), mitochondrial membrane potential (TMRE fluorescence intensity detected by PE channel), or mitochondrial superoxide level (MitoSOX red fluorescence intensity detected by PE channel) were performed on a BD LSR II Flow Cytometer (Becton Dickinson, San Jose, CA). Data are presented as histograms for, and as bar graphs of average signal intensity of ~50,000 ungated events.

Statistical Analysis
Data are reported as mean ± SEM, unless otherwise stated. Unpaired Student's *t* test or ANOVA with Tukey's comparison were used for paired and group comparisons, respectively. $P<0.05$ was defined as significant.

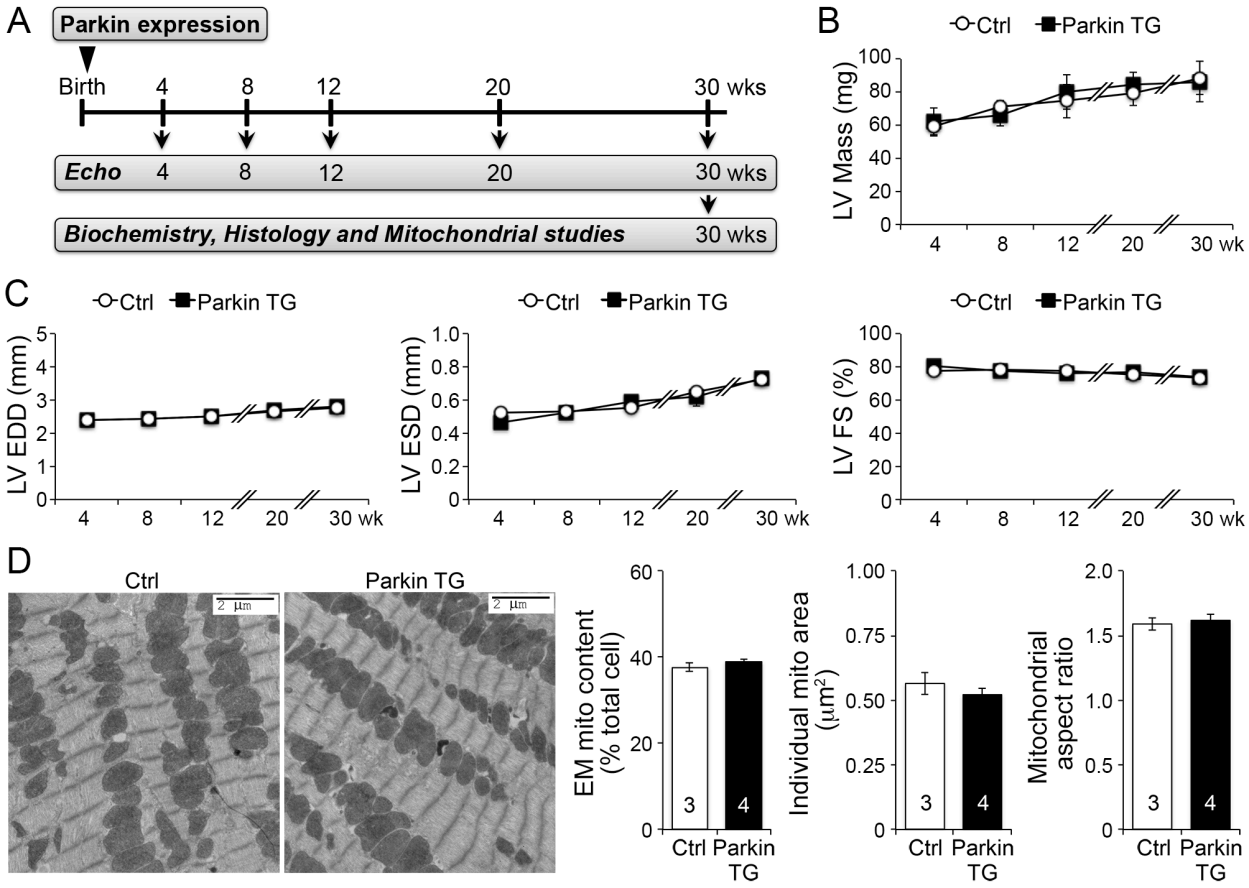
Supplemental Figures and Figure Legends



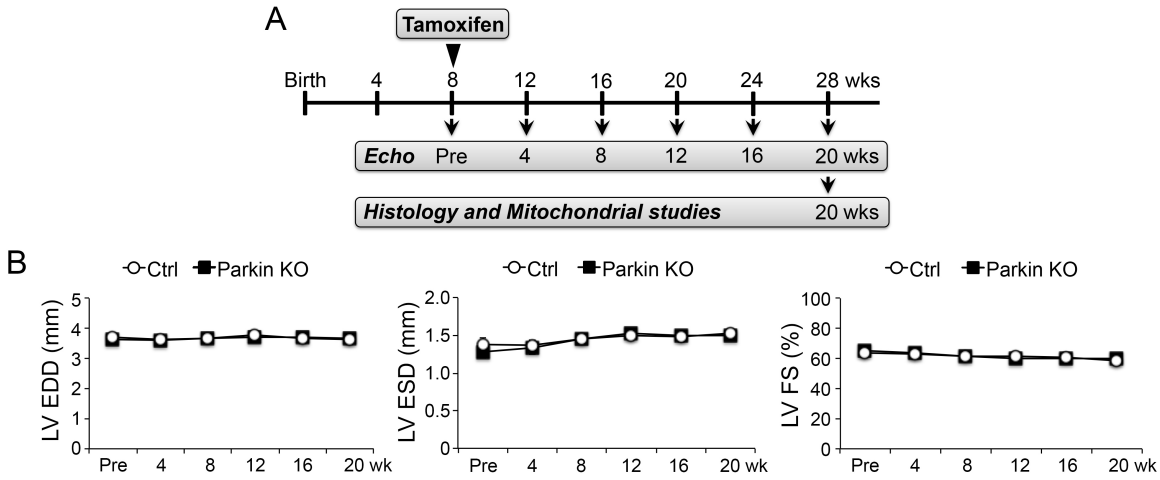
Online Figure I. Ultrastructural examination of cardiomyocyte mitochondria in Drp1 null hearts. Representative transmission electron microscopic images showing loss of cardiomyocyte mitochondria 6 weeks after conditional cardiac Drp1 deletion in an 8 week old mouse. Original magnification is 1,000x (upper left) and 3,000x (upper right). For clarity, the areas in the black squares of the upper right panel are enlarged in the lower panels.



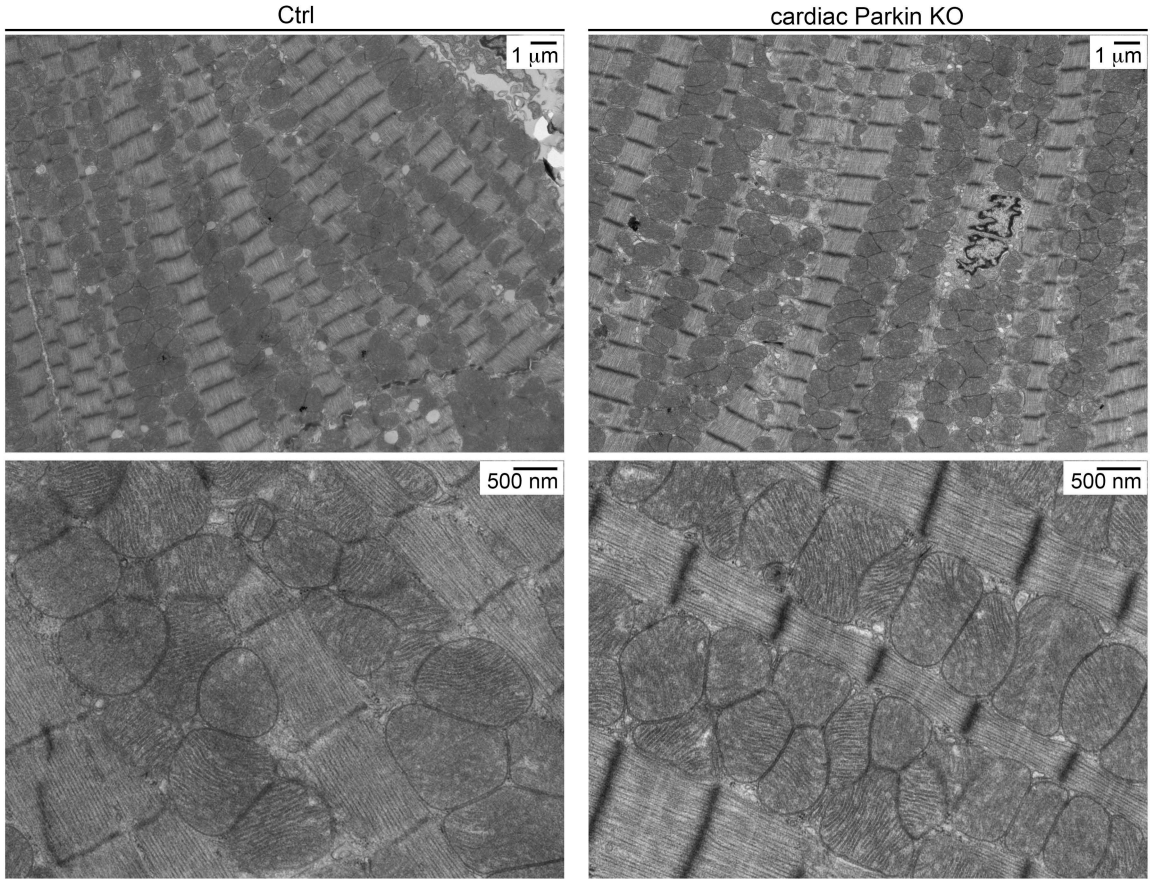
Online Figure II. Multi-antibody immunoblot analysis of Parkin in the 100,000 x*g* supernatant myocardial fraction of normal, cardiac Parkin-deficient, and cardiac Drp1-deficient mouse hearts. GAPDH is loading control; arrows indicate predicted position of Parkin or GAPDH protein.



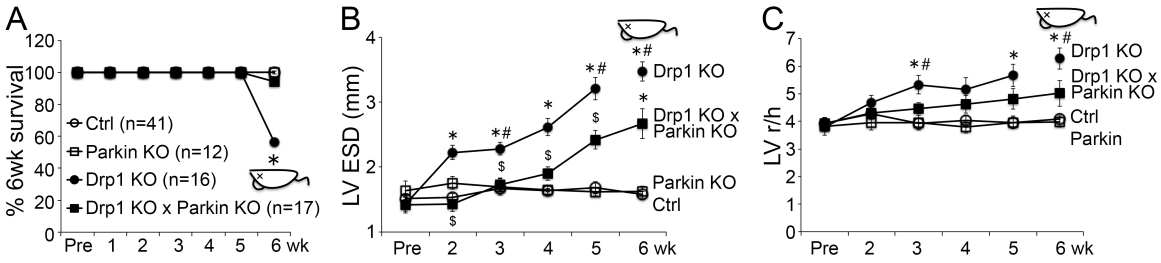
Online Figure III. Serial studies of *myh6*-Parkin cardiac transgenic mice. **A**. Schematic depiction of experimental design. **B-C**. Serial quantitative group echocardiographic data showing left ventricular (LV) mass, LV end-diastolic dimension (EDD), LV end-systolic dimension (ESD) and fractional shortening (FS) from 4 to 30 weeks. Ctrl (nontransgenic litermates, n=5) are open circles; Parkin transgenic mice (n=5) are closed squares. **D**. Representative transmission electron microscopic images showing cardiomyocyte mitochondria of 30 week old mouse hearts. Original magnification is 3,000x. Quantitative metrics for mitochondria are to the right. Data are shown as mean ± SEM. No statistically significant differences are present.



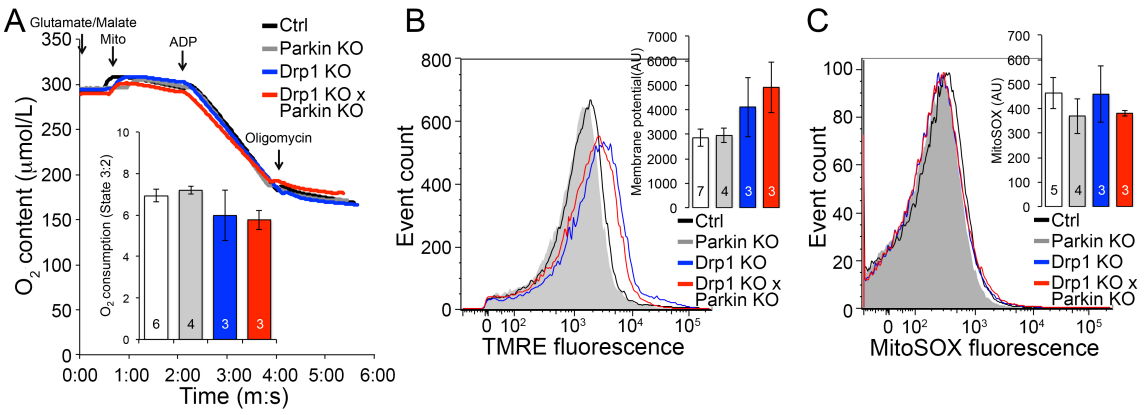
Online Figure IV. Serial studies of conditional cardiac Parkin knockout mice. A. Schematic depiction of the experimental design. **B**. Quantitative group echocardiographic data showing LV EDD, ESD and FS before, and up to 20 weeks after, cardiac Parkin ablation. Controls (Ctrl, mice with floxed alleles only, n=12) are open circles; cardiac Parkin knockout mice (n=10) are closed squares; data are shown as mean ± SEM. No statistically significant differences are present.



Online Figure V. Ultrastructural examination of cardiomyocyte mitochondria in cardiac Parkin-deficient hearts. Representative transmission electron microscopic images showing cardiomyocyte mitochondria of 14 week old mouse hearts, 6 weeks after conditional cardiac *Park2* ablation. Controls (Ctrl) are mice with floxed alleles only. Original magnification is 3,000x (upper panel) and 5,000x (lower panel).



Online Figure VI. Survival and cardiac function of mice after conditional cardiomyocyte-specific Drp1, Parkin, and combined Drp1/Parkin ablation. **A**. Survival of mice after tamoxifen-mediated gene recombination at 8 weeks of age. **B**. Quantitative group serial echocardiographic data showing LV ESD and the ratio of LV end-diastolic radius (r) to wall thickness (h) before, and up to 6 weeks after, Drp1, Parkin, and combined Drp1/Parkin gene ablation. Controls (Ctrl, floxed alleles without Cre, n=16) are open circles; cardiac Parkin knockout mice (n=5) are open squares; cardiac Drp1 knockout mice (n=16 for the time points before 6 weeks; n=9 for the 6-week time point) are closed circles; concomitant cardiac Drp1 and Parkin knockout mice (n=9) are closed squares. The dead mouse icons indicate that cardiac function at 6 weeks reflects only live mice, and not the ~half of cardiac Drp1 KO mice that died between 5 and 6 weeks after gene recombination. Data are shown as mean ± SEM; * $p<0.05$ vs Ctrl and # $p<0.05$ vs Drp1 KO mice.



Online Figure VII. Functional studies of cardiac Drp1 KO, cardiac Parkin KO, and cardiac Drp1/Parkin KO mouse heart mitochondria. **A**. Isolated cardiac mitochondrial respiration at 6 weeks after gene ablation; inset is quantitative group data for state 3 (ADP stimulated)/state 2. **B**. Flow cytometry analyses of isolated cardiac mitochondrial transmembrane electrical potential measured with TMRE. **C**. O_2^- measured with MitoSOX Red. Insets for B and C are quantitative group data. White bar (black line) is Ctrl, grey is Parkin KO, blue is Drp1 KO, and red is Drp1 KO/Parkin KO. Data are mean ± SEM of indicated number of experiments. There are no statistically significant differences.

Supplemental References

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